Attraction and Resistance in the *Picea abies* – *Ips typographus* System

Host Choice in the Eurasian Spruce Bark Beetle

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Abstract

Decades of research have gathered detailed knowledge about the Eurasian spruce bark beetle (*Ips typographus* L.) and its interaction with its host tree Norway spruce (*Picea abies* (L.) H Karst). The strong pheromone attraction enabling this beetle to kill healthy host trees is a well known process, and pheromones are widely used to trap beetles in forest pest management. Yet, host finding and final host choice by the first attacking pioneer beetles is only vaguely understood. The aim of this thesis was to identify olfactory cues for host choice from habitat to final colonisation.

The importance of non-host volatiles (NHV) and old-host volatiles (OHV) for habitat finding, indicating unsuitable habitats, was confirmed in a large scale field experiment at forest edges in Slovakia and Sweden. A blend of NHV/OHV dispensers created an artificial semiochemical diversity, and reduced attacks in treated versus untreated plots. An active inhibitory range of ≈15 m was determined for the protection. A detailed study of host bark chemistry in relation to natural bark beetle attacks showed the importance of induced host defence capacity for resistance in *P. abies* against *I. typographus* attacks. The only individual host compound that seemed to influence host choice in this study was 1,8-cineole. Volatiles that were collected from bark of felled and standing healthy trees were tested on *I. typographus* antennae by using combined gas chromatography-electroantennographic detection. The electrophysiological studies also including single sensillum recording technique revealed eight novel ligands of host compounds, of which six are oxygenated, eliciting strong responses in olfactory sensory neurons. Proportional increase of oxygenated host compounds in host chemistry were shown to be related to induced defence and decay of host bark, and are proposed as semiochemicals for host evaluation by *I. typographus*. Quantification of host volatiles released by felled, healthy, and naturally attacked standing trees indicate that the onset of beetle attack correlated with high amounts of released host volatiles. Field trapping experiments and laboratory no-choice feeding assays with a subset of the oxygenated compounds confirmed the inhibiting activity of 1,8-cineole, and indicated both negative and positive effects on pheromone attraction and feeding by other antennally active compounds.

Keywords: habitat recognition, non-host volatiles, semiochemical diversity, induced defence, phenolics, terpenes, oxygenated host compounds, methyl jasmonate, GC-EAD, olfactory sensory neurons, feeding assay

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Populärvetenskaplig sammanfattning

Genom många årtionden av forskning har det uppnåtts en detaljerad bild av biologi och ekologi hos granbarkborren (*Ips typographus*) och dess värdväxt gran (*Picea abies*). Trots detta är kunskapen om hur granbarkborren lokaliserar och slutligen väljer en lämplig gran för angrepp och reproduktion mycket bristfällig. Ett starkt feromonsystem möjliggör att en liten insekt kan övermanna och döda en stor gran, som har ett utvecklat försvarssystem i form av kåda och andra fysiologiska mekanismer. Feromonet utsöndras av de först anlända hannarna, så fort de börjar gnaga i barken. Tusentals andra hannar och honor lockas av feromon till samma träd inom några få dagar, som normalt inte överlever ett sådant koordinerat angrepp. De kemiska ämnen, som tillsammans utgör grandoften, kan tänkas att spela en viktig roll för de s.k. pionjärerna, som inte kan följa ett feromonspår, för att hitta en gran. De behöver också kunna avgöra granens försvarsförmåga, då en försvagad gran innebär en mycket mindre risk för en ensam hanne. En ensam angripare kan troligtvis inte veta i förväg hur många andra barkborrar, som kommer att hörsamma hans ”rop på hjälp” i form av feromonsignalen. Man vet också av erfarenhet att försvagade granar och särskilt vindfällda träd är prioriterade mål, framför allt innan en population har uppnått epidemisk storlek. Dock har det hittills inte varit möjligt att i försök tydligt visa en attraktion av granbarkborrar till grandoft. Detta trots att neurofysiologiska studier av barkborrarnas luktnerver visar, att de har möjlighet att fysiologiskt kunna reagera på specifika ämnen, som är del av doften. Ämnena binder till specifika doftreceptorer, som finns i nervcellers membran. Hundratals dofthår på insektens antenner innehåller minst två luktnerver var (Bild 1). Varje receptor svarar specifikt på enskilda kemiska ämnen eller på ett mindre antal olika ämnen, så att en elektrisk nervsignal bildas, som i hjärnan moduleras tillsammans med andra inkommande signaler och eventuellt utlöser ett adekvat beteende.

I avhandlingens första studie undersöktes hur doftsignaler från icke-värdväxter hjälper barkborrarna att undvika olämpliga lokaler och hitta sin värdväxt. Denna effekt kan tillämpas i skadebekämpningen genom att dispensrar, som utsöndrar en blandning av repellerande dofter, hängs på utsatta granar, såsom nya skogskanter, och fördröjer angreppet. Studien visade en tydlig reduktion av angrepp på skyddade granar vid hög populationsstorlek. Vid lägre populationsstorlek visades en skyddseffekt av ≈15 m avstånd från doftpåsarna på en grupp av 10 granar. För en effektiv användning i skadebekämpningen behöver dispensrarna kombineras med feromonfällor för att kunna fånga bort lokala populationer på våren innan barkborrarna börjar förflytta sig över längre avstånd.

I den andra studien undersöktes barkens kemi hos granar i ett område med stora barkborreproblem. För att i förväg undersöka varje grans förmåga att försvara sig, mobiliserades det kemiska försvaret i varje gran i undersökningen lokalt på en punkt i stammens nedre del genom att behandla barken med ett växthormon. Ett antal av de undersökta granarna angreps senare under sommaren. Det visade sig att granarna, som hade blivit dödade, tydligt skilde sig i sin kemiska försvarsförmåga från granar som överlevde. Däremot var skillnaderna i den kemiska profilen av bark, där försvaret inte hade inducerats, mycket små. Endast mängden av ämnet 1,8-Cineol skilde sig i den oinducerade barken. Dödade granar hade mycket låga halter, medan granar, som

överlevde angreppet hade signifikant högre halter. Detta ämne kan vara en viktig markör för angripande hannar, att trädet är försvagat. Alternativt kan det tänkas motverka feromonattraktionen hos andra barkborrar. Studien understryker i huvudsak betydelsen av balansen mellan trädens försvarsförmåga och barkborrepopulationens storlek för förloppet av ett barkborreutbrott.

I den tredje studien samlades doftprover från barken av fällda träd, friska stående träd och stående träd, som just hade börjat att angripas. Med hjälp av elektrofysiologiska metoder (Bilder 2 och 3) undersöktes barkborreantenners nervsvar på ämnen från de insamlade doftproverna. Förutom nervsvar till huvudbeståndsdelar i grandoften, som redan var kända för att utlösa elektriska nervsvar, hittades relativt mycket kraftigare nervsvar till ett antal ämnen, som förekommer endast i mycket små kvantiteter i avdunstningen från granbarken. Dessa ämnen är huvudsakligen oxygenerade terpener, ämnen som omvandlas från huvudbeståndsdelarna i kådan bl. a. vid mikrobiell nedbrytning eller vid kontakt med luft. Den kemiska undersökningen visade, att en del oxygenerade ämnen i barken eller i avdunstningen ökar proportionellt vid inducering av försvarsmekanismer och vid barkens åldrande. Det är därför troligt, att dessa ämnen är viktiga för barkborrarnas förnimmelse av trädens status. Kvantifieringen av avdunstningarna pekar på, att starten av angreppen sammanföll med en högre mängd av avgivna ämnen både i fällda och angripna stående träd.

Den sista studien försökte att hitta ett beteende relaterat till de oxygenerade ämnena. Ett inledande fältexperiment testade om oxygenerade ämnen kan utöva attraktion åtminstone på en liten del av barkborrar tillsammans med grandoftens huvud-beståndsdelar. Den valda blandningen i olika doser lyckades dock inte fånga barkborrar i större antal än en obetad fälla. I ett annat fältexperiment användes feromoner i låg dos som attraktionsmedel, och testämnena tillsattes i en låg och en högre dos, som båda motsvarade naturligt förekommande avdunstningsnivåer från träd. Ämnet 1,8-Cineol reducerade attraktionen signifikant i båda doser. I en annan avhandling har det visats att nervceller, som svarar på 1,8-Cineol, minskar nervsvaret i en granncell, som specifikt svarar på en essentiell feromonsubstans. Även några andra ämnen minskade attraktionen mot feromon något, medan ämnet Karvon ökade attraktionen lätt. Utöver fältförsöken gjordes det gnagförsök i laboratorium (Bild 4). Barkborrar sattes i glasrör, som var fyllda med ett konstgjort barksurrogat. Testämnen tillsattes i tre låga, naturliga koncentrationer och längden av det konsumerade mediet mättes och jämfördes mot en kontroll utan testämne. Hannar reagerade tydligare på testämnena än honor. Även här hade Karvon en svagt positiv effekt på barkborrarna, medan flera av de övriga ämnen minskade baggarnas gnag något, dock bara i den lägsta och mellersta dosen.

Sammanfattningsvis visade denna avhandling på betydelsen av både värdväxt- och icke-värdväxtämnen för barkborrarnas orientering. Dock är det fortfarande ovisst vilka specifika beteenden, som utlöses av värdväxtämnen, som kan uppfattas av granbarkborrens antenner. Flera nya ämnen har upptäckts vara betydelsefulla för barkborrarnas uppfattning av sin värdväxt. Granens resistens verkar huvudsakligen vara styrd av dess förmåga att effektivt inducera sitt kemiska försvar.

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Schiebe C, Blaženec M, Jakuš R, Unelius CR, Schlyter F (2011). Semiochemical diversity diverts bark beetle attacks from Norway spruce edges. *Journal of Applied Entomology* 135:726-73.

II Schiebe C, Hammerbacher A, Birgersson G, Witzell J, Brodelius P, Gershenzon J, Hansson B, Krokene P, Schlyter F (2012) Inducibility of chemical defenses in Norway spruce bark is correlated with unsuccessful mass attacks by the spruce bark beetle. *Oecologia* 170:183-198.

III Christian Schiebe, Göran Birgersson, Julia Jankuvová, Peter E. Brodelius, Fredrik Schlyter, Bill S. Hansson. Strong antennal responses by the bark beetle *Ips typographus* L. (Scolytidae, Scolytinae) to stress related host compounds (manuscript).

IV Christian Schiebe, Julia Jankuvová, Miroslav Blaženec, Rastoslav Jakuš, Fredrik Schlyter. Attraction and feeding behaviours modulated by stress related oxygenated host compounds at natural doses in a bark beetle (manuscript).

The contribution of Christian Schiebe to the papers included in this thesis was as follows:

I Chose and established experiment sites and performed all field work for Swedish experiments and analysed the data in collaboration with Fredrik Schlyter. Wrote the paper in collaboration with co-authors.

II Developed the initial idea together with the co-authors. Did sample and data collection in collaboration with field assistants and all analyses for terpene chemistry. Did all data analysis and wrote the paper together with the co-authors.

III Developed the initial idea together with the co-authors. Developed the sampling method together with Göran Birgersson. Collected aerations in field and did all other field work. Analysed the samples. Carried out all electrophysiological studies, analysed them and wrote the manuscript in collaboration with the co-authors.

IV Planned, instructed and did part of behavioural studies. Evaluated data and wrote the manuscript together with Fredrik Schlyter.

Abbreviations

Ci	1,8-Cineole
cV	*cis*-Verbenol
GC-EAD	combined Gas Chromatography-ElectroAntennographic Detection
GC-MS	combined Gas Chromatography – Mass Spectrometry
MeJ	Methyl Jasmonate
MB	2-Methyl-3-Buten-2-ol
NHV	Non Host Volatiles
OHV	Old Host Volatile
OSN	Olfactory Sensory Neuron
PP-cell	Polyphenolic Parenchyma cell
SSR	Single Sensillum Recordings
TRD	Traumatic Resin Duct

1 Objectives

The objectives of this study were to elucidate the semiochemical mechanisms for host choice in the Eurasian spruce bark beetle *Ips typographus* (L.). The studies included beetle habitat recognition and the applied aspects of semiochemical diversity for pest management, host chemistry influencing host choice and host resistance, and neurophysiological and behavioural aspects of host recognition. Special emphasis was put upon host compounds indicating host vitality and their role as markers for acceptance and resistance in the sensory system of the beetle. An additional perspective was to find indications for primary attraction of pioneering beetles that attack trees without the involvement of pheromonal attraction.

2 Introduction

2.1 Primary and secondary attraction in bark beetles

Bark beetles (Coleoptera) belong to the superfamily Curculionoidea and are mainly consumers of dead wood. Some species, also called ambrosia beetles, from the subfamilies Platypodinae and Scolytinae live in symbiosis with different fungi that enable them to break down wooden materials. These species only colonise dead wood or damaged trees, and are attracted to the breeding material by kairomones (host stimuli) alone, a process referred to as "primary attraction". In the Scolytinae, several species in the genera *Dendroctonus, Ips, Scolytus, Pseudohylesinus,* and *Pityogenes* are often termed to be "aggressive" by their capacity to kill and colonise living trees (Wood, 1982b). These bark beetles oxygenate host compounds and release them as pheromones that attract conspecifics, but in many cases also *de novo* produced pheromones are known. These pheromones serve both as sex pheromones attracting mates, and as so called aggregation pheromones attracting both sexes (Wood, 1982a; Schlyter & Birgersson, 1999; Seybold *et al.*, 2000). Aggregation pheromones coordinate mass attacks on single or groups of healthy trees, depleting host defences and killing the trees. Attraction to the host by pheromones is called "secondary attraction", and is coupled to host chemistry only by the conversion of pheromone components from host precursors. Also enhancement of pheromone attraction by host compounds is well described in several systems (Schlyter & Birgersson, 1999; Seybold *et al.*, 2006). Weather pioneering beetles (the beetles initiating an attack before the existence of a pheromone plume) in tree killing species select suitable trees after random landing or by means of primary attraction in tree killing species is, however, a matter of debate (Moeck *et al.*, 1981; Gries *et al.*, 1989; Byers, 1996; Baier *et al.*, 1999). Some species of *Dendroctonus* and *Ips*

have been shown to be attracted to single host monoterpenes (Seybold *et al.*, 2006), but there is only weak evidence that *I. typographus* is attracted to host material or single host compounds (Austarå *et al.*, 1986; Hulcr *et al.*, 2006).

2.2 Host preferences in *Ips typographus* and pest status

The Eurasian spruce bark beetle exhibits a tree killing behaviour only during outbreaks, after strong population increase following storm felling and/or periods of severe drought or in stands severely stressed and weakened by other factors. Under the more common endemic conditions the beetles are confined to reproduce in still fresh bark of sporadically occurring windfalls or damaged trees, but cannot live on dried bark or dead wood. A sudden supply of broken trees offers an almost limitless resource of breading material for small populations, which generally results in low attack densities and high reproductive success (Botterweg, 1983; Komonen *et al.*, 2011). With favourable weather conditions following a storm event and without sufficient removal of windfalls, an outbreak of the population is generally inevitable. After a couple of generations the population reaches the threshold where the defence of standing trees can be overcome (Mulock & Christiansen, 1986; Schroeder & Lindelöw, 2002; Økland & Bjørnstad, 2006). In the incipient phase of population growth, however, stressed standing trees still are the preferred attack focus, and the infestation spreads along to neighbouring trees for as long as bark beetles are attracted to the emanating pheromone plume (Schlyter & Anderbrant, 1989). In later phases of population growth, standing trees are colonised with higher densities than fallen trees (Komonen *et al.*, 2011) that also may remain unattacked (Schiebe, personal observations), corresponding to patterns shown by *Dendroctonus ponderosae* Hopkins in *Pinus contorta* Douglas var. *latifolia* (Boone *et al.*, 2011). In extreme situations very high *I. typographus* populations also rarely attack Scots pines (*Pinus sylvestris* L.) (Komonen *et al.*, 2011). In Sweden, *I. typographus* has killed a volume of 9 million m^3 of Norway spruce trees in three large outbreaks during the last 50 years, compared to 600 million m^3 of Lodgepole pine killed by *D. ponderosae* in British Columbia within the same period (Kärvemo & Schroeder, 2010).

2.3 The system – a part of the community

This thesis assesses the interactions between a bark beetle and its host. But it should not be forgotten that many interactions in this system involve a still unknown number of organisms. A relatively well studied part in this

community, with a major impact upon the system, are fungal species vectored by the beetles (mainly ophiostomatoid fungi), possessing different virulence for the host (Krokene & Solheim, 1996; Krokene & Solheim, 1998; Jankowiak, 2005; Sallé *et al.*, 2005b). The beetle-fungus symbiosis is clearly obligate for the fungus, but seems to be more facultative for the beetle, as the correlation between a successful colonisation by the beetle and the virulence of the vectored fungi is not always clearly determined (Solheim, 1992; Lieutier *et al.*, 2009; Six & Wingfield, 2011). Not only obligate fungus – beetle associations with virulent impact upon the tree, also fungi infecting beetles and antagonistic fungus-fungus interactions are part of this community (Solheim, 1992; Klepzig & Six, 2004; Jankowiak, 2005). In addition, symbiotic and antagonistic bacteria may impact fungus establishment and beetle colonisation (Adams *et al.*, 2009; Popa *et al.*, 2012). A more obvious part of the community are competing bark beetle species and a great diversity of predators and parasites (Weslien, 1992).

2.4 Host quality and host defences

The suitability of a tree as a resource for beetle reproduction is defined by two main factors: the vigour of the tree and beetle population dynamics (Paine *et al.*, 1984; Mulock & Christiansen, 1986). The intricate balance between these two factors defines the threshold of successful attack that determines weather the beetles will be able to overcome host defences. A third factor is of major importance: beetles choose bark with high quality bark, given the possibility to survive in a living, defending host. Also in undefended, broken trees, larger diameter trees are preferred over smaller trees (Göthlin *et al.*, 2000). When attacking standing trees, large trees and mature forest is the preferred choice (Wermelinger, 2004). In *Ips pini* (Say) reproductive performance was positively related to different indices of tree vigour in colonised, cut jack pine trees (*Pinus banksiana* Lamb.) (Reid & Robb, 1999). On the other hand tree vigour is also positively related to the threshold of successive attack, i.e. more beetles are needed to kill a tree with high vigour and high quality phloem (Mulock & Christiansen, 1986).

Conifers possess several anatomical and chemical strategies for their defence. The outer bark (periderm) provides a physical barrier, that protects the vital assimilate transport within the nutrient and energy rich phloem and the thin and undefended meristematic cambium layer. This barrier includes tight layers of mostly dead cork cells with lignified and suberized walls and sharp calcium oxalate crystals that also can be found in the living phloem, and may function as hinders against boring and chewing (Hudgins *et al.*, 2003).

Lignified stone cells (sclereids) form clusters containing mostly lignin, but even phenolics (Franceschi *et al.*, 2005; Li *et al.*, 2007) that can have antifungal and antifeedant properties (Brignolas *et al.*, 1998; Evensen *et al.*, 2000; Faccoli & Schlyter, 2007). In the secondary phloem large quantities of phenolics are deposited in the vacuoles of so called polyphenolic parenchyma cells (PP-cells). The PP-cells form annual tangential rings and can be discerned like the annual rings in the xylem for decades (Krekling *et al.*, 2000; Franceschi *et al.*, 2005).

Upon wounding, trees exude resin, which is the most conspicuous defence in the Pinaceae. In *Picea abies* the resin producing cells form tube-like ducts in both xylem and phloem, where the resin is synthesized in the epithelial cells, lining the cavities within these structures, and is extruded and stored there under pressure (Nagy *et al.*, 2000). The resin is formed by volatile monoterpenes (10 carbon), semi-volatile sesquiterpenes (15 carbon) and the 20 carbon diterpenoid acids. After volatilisation of the mono- and sesquiterpenes the crystallised diterpenes seal the wound and build up a mechanical barrier against intruders. The preformed (constitutive) defence ability is dependent on the amount of stored oleoresin, but also on it's chemical composition and on the toxic effect to herbivores. Both ray cells and resin ducts are involved in the translocation of resources between phloem, sapwood and heartwood (Berryman, 1972), and therefore the resin produced in the xylem is able to extrude into the phloem.

In addition to constitutive defences, most healthy trees are able to induce a second barrier of defence mechanisms upon challenge. Existing pathways, like the phenylpropanoid pathway (phenolics) and the isoprenoid pathway (terpenoids) can be induced to rapidly produce more of defensive compounds, and also to alter the composition of the extruded oleoresin to increase its toxicity towards invasive organisms (Martin *et al.*, 2002). The cambium layer can generate new anatomical structures like traumatic resin ducts (TDs) that increase the volume of resin ducts, and contribute to acquired resistance against future challenges. Swelling and activation of PP-cells is another visible sign for an induced defence reaction. Together with the formation of TDs, the PP-cells have been shown to be associated with increased resistance to pathogenic fungi (Krokene *et al.*, 2003).

2.5 Olfactory recognition

Olfactory host recognition in *I. typographus* is difficult to establish in behavioural experiments. Several studies indicate, however, primary attraction to natural spruce material or the modulation of secondary attraction by

synthetic host compounds or natural odours (Austarå *et al.*, 1986; Lindelöw *et al.*, 1992; Reddemann & Schopf, 1996; Franklin *et al.*, 2000). It is well known, that the olfactory system of the beetle is able to respond to several constituents of host odour, but the role of single compounds for host attraction is still unknown. Beetle antennae possess a similar number of olfactory sensory neurons (OSN) that are tuned to host compounds, non-host compounds, and intraspecific pheromones or to pheromones of competing species (Dickens, 1978; Tømmeras & Mustaparta, 1987; Andersson *et al.*, 2009). The ventral side of the antennal club of the beetles is covered with sensilla, with the highest density in two undulating bands in the middle of the segment and a third area on the distal part of the club (Fig. 1). Generally each of the sensilla are innervated with two OSNs (Hallberg, 1982). The binding of odorant molecules (called ligands) to receptor proteins, situated in the dendritic membranes of the neurons, triggers molecular mechanisms within the cell eventually eliciting an electric signal along the neuron. The neuronal signal propagates through the neuron and converge with signals from other neurons in the antennal lobe. A modulated signal may finally result in a behaviour (Hansson & Stensmyr, 2011). Consequently, it is impossible to predict a behaviour from the recorded electrophysiological response alone.

The behaviour related to *I. typographus* pheromone components 2-methyl-3-buten-2-ol (MB), *cis*-verbenol (cV), ipsdienol and ipsenol is well established (Schlyter *et al.*, 1987a; 1987b; 1987c). Also the antennal response elicited by several compounds found in non-hosts has been shown to result in behavioural avoidance of non-host signals in *I. typographus* (Zhang & Schlyter, 2004; Schiebe, 2006). Only few indications exist for a behaviour related to single host compounds (Reddemann & Schopf, 1996; Hulcr *et al.*, 2006). So far, a clear behavioural effect of a host monoterpene has only been demonstrated for (–)-α-pinene, synergistically enhancing the attraction to very low pheromone doses (Erbilgin *et al.*, 2007). The inhibitory effect of the oxygenated product of (–)-α-pinene, (–)-verbenone is, in contrast, well established as a signal indicating an overexploited, unsuitable host (OHV: old host volatile) (Bakke, 1981; Leufvén *et al.*, 1984; Schlyter *et al.*, 1989).

3 Aims and methods

3.1 Host choice on habitat scale

Assessing the orientation of small insects towards a suitable host habitat in a landscape is a complex task. The importance of non-host volatiles (NHV) and semiochemical diversity for the behaviour of *I. typographus* had been shown previously by the strong inhibition exerted by NHV on beetle attraction to pheromone traps (Zhang & Schlyter, 2003; Zhang & Schlyter, 2004; Schiebe, 2006) and by pair wise choice/non choice tests of treated/untreated forest sections that showed reduction in the number of attacked trees in treated sections (Jakuš *et al.*, 2003). Because of the difficulties in predicting local and regional bark beetle population sizes and movements, it is hard to predict the probabilities of natural attacks within a particular stand. To obtain significant results large scale experiments with sufficient replication are needed.

- The aim of the first study (Paper I) was to obtain more evidence for the repellent effect of NHV and OHV on dispersing beetles on a habitat and landscape scale.
- Methods: We examined attack patterns in the previous years within localities in order to predict the probability of attacks. Several localities with high attack probability on a regional scale were chosen in Sweden and Slovakia. The design of treatments (protected and unprotected control plots) was similar in all localities. In Sweden attack patterns around treated plots were assessed on landscape scale by GPS-positioning and treatment of data by geographic information system (GIS).

3.2 Discrimination between host trees

Do chemical profiles of chosen or non chosen trees guide beetle discrimination between individual trees? In order to answer this question it is necessary to assess differences of the chemical profile between trees before colonisation occurred. No artificial pheromones should be applied to attract beetles. A second important question is, whether there are differences between trees when responding to beetle attacks.

- The aim of the second study (Paper II) was to determine the chemical profiles of trees prior to attack in an area with an epidemic beetle population and with a high probability for attacks. As many chemical compounds as possible were to be included in the study. In addition, also the induced defence capacity of each tree should be assessed.
- Methods: Given a high probability of beetle attacks within a stand, it is, nevertheless, impossible to predict where and to which extent future attacks will occur. Consequently we had to sample a large number of trees at exposed places before beetle flight started. To assess the induced defence capacity of individual trees we applied local treatments with the phytohormone methyl jasmonate (MeJ), which is known to elicit defence reactions close to the application point, without changing the entire trees defence status. Samples were analysed by combined gas chromatography – mass spectrometry (GC-MS) for analysis of terpenoid compounds and with liquid chromatography-mass spectrometry with electrospray ionization (LC–ESI–MS) for analysis of phenolic and alkaloid compounds. Anatomical defence structures were studied by microscopic analysis of cross-sections of phloem and cambium layer. To evaluate differences in the chemical profile between trees, that after sampling were attacked, not attacked or resisted attack, we applied multivariate analysis.

3.3 Which host compounds can beetles smell?

Electrophysiological studies of *I. typographus* olfaction, so far, were designed to exclusively test synthetic host compounds, and tests of natural volatiles were not able to resolve OSN-responses to the level of individual compounds. So far, the whole bouquet of host compounds in natural host volatiles has not been tested. It may contain more compounds eliciting responses on beetle antennae, than previously tested by synthetics. In addition, are there differences between volatiles from weakened trees and healthy trees concerning compounds eliciting responses?

- The aim of the third study (Paper III) was to obtain as many odour collections as possible throughout the progress of a beetle attack on

weakened host trees in order to detect eventual volatile markers for host suitability. We also wanted to compare these collections to collections from unattacked healthy trees. To be able to quantify aerations, collections should be achieved by a standardised method. Aeration samples should be tested by both chemical analyses and for their biological activity.

- Methods: Volatiles from felled and standing healthy trees were collected on an adsorbing polymer and eluted with solvent. Collected samples were tested with combined gas chromatography – electro electroantennographic detection (GC-EAD) (Fig. 2). Chemical analysis of samples was performed with GC-MS. Identified compounds were further tested on single sensilla on beetle antennae using single sensillum recording technique (SSR) (Fig. 3) in order to localise responding OSNs.

3.4 Behaviour of compounds with antennal activity

The difficulties to establish the role of host monoterpene hydrocarbons in the host choice behaviour of *I. typographus* may be related to the ecological importance of specific ratios of ubiquitous compounds (Bruce *et al.*, 2005). The possible importance of minor constituents of host odour for eliciting a relevant behaviour may be another, previously neglected factor. The detection of minor constituents with strong antennal activity may thus be the missing link needed to demonstrate behavioural activity of individual compounds or synthetic host odour bouquets.

Wind tunnel experiments are a common tool in behaviour tests of insect olfaction. Hovewer, since the flight behaviour of bark beetles include large vertical movements (Choudhury & Kennedy, 1980) this tool is not applicable for *I. typographus*. Field experiments are dependent on a sufficiently large population size and are only easily applicable when testing the modulation of pheromone attraction. Walking bioassays in laboratory suffer from the beetles' tendency to hide, when they are exposed on open surfaces.

- The aim of the fourth study (Paper IV) was to find behaviours related to a number of new host compounds with antennal activity. A first test of a host volatile bouquet was also included.
- Methods: We used field trapping experiments with and without pheromones. Beetle feeding was assessed in no-choice feeding experiments in laboratory using artificial medium with single beetles (Fig. 4).

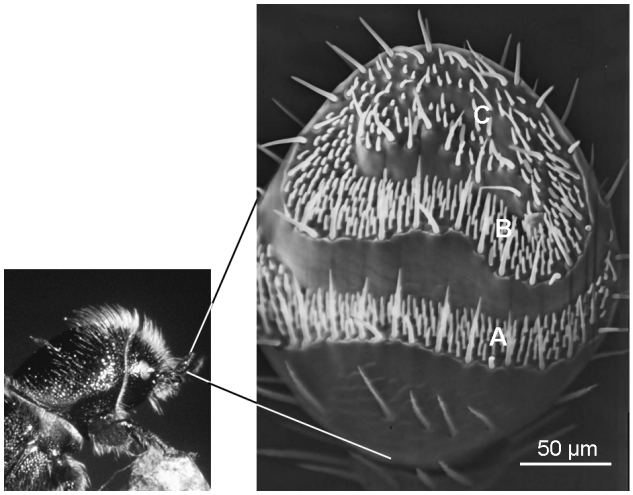


Figure 1. The ventral side of the antennal club of the *Ips typographus* antenna. Olfactory sensilla are present in two undulating bands (A and B) and in a distal area (C).

Left photograph Göran Birgersson, right photograph Eric Hallberg.

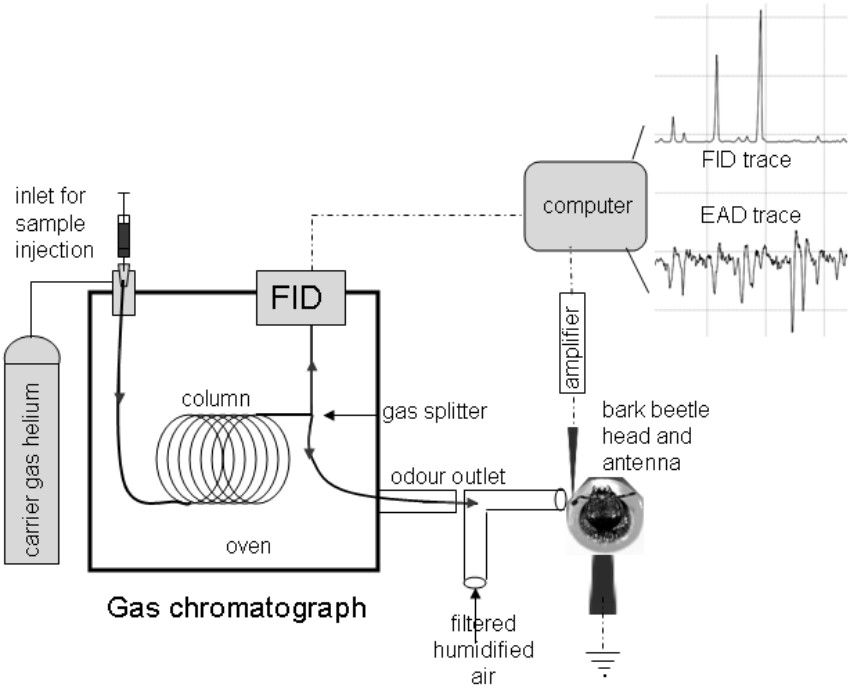


Figure 2. Schematic drawing of a GC-EAD device. FID (flame ionization detector) traces single compounds separated from the sample mixture by the GC-column, simultaneously as they are presented to the beetle antenna. The EAD measures the drop of electric potential at the antenna when any OSNs are elicited by a particular compound. Also very small amounts beyond the detection level of the FID can elicit a response of the antenna.

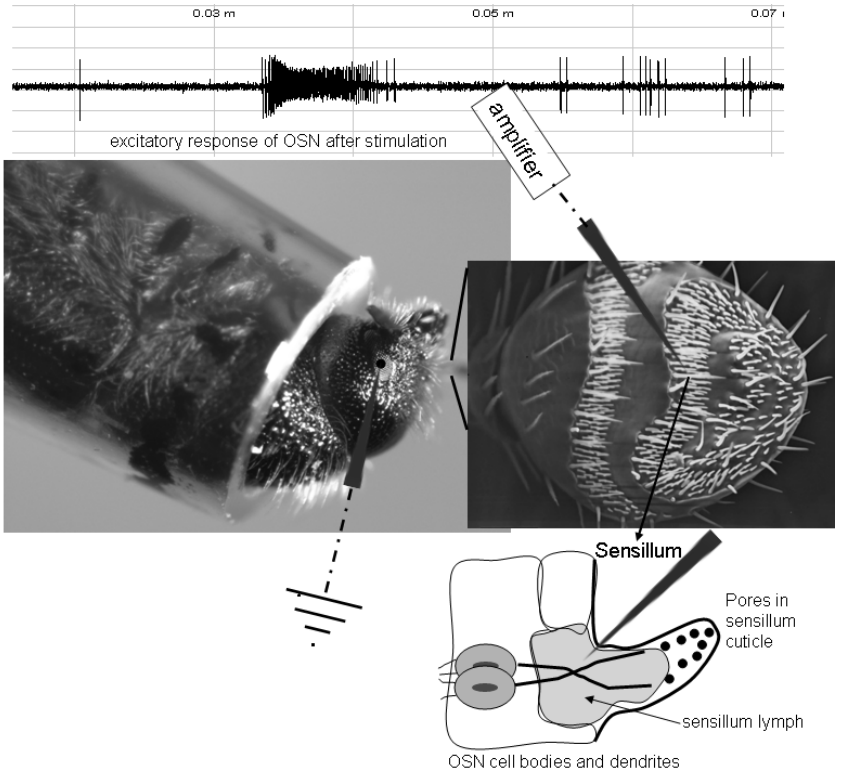


Figure 3. In SSR electric potentials are measured that arise in the hemolymph of a single sensillum, when a particular compound elicits a response within one of the two present OSNs. Left photograph E. Hatano, right photograph E. Hallberg.

Figure 4. One choice feeding assay with individual beetles feeding on an artificial medium amended with the compound to be tested. Photograph F. Schlyter.

4 Habitat recognition (Paper I)

4.1 Semiochemical diversity in mixed forests

Our present even-aged and mono cultural forest structures cannot be imagined as the natural environment for the evolution of conifer – bark beetle adaptions. In contrast, Norway spruce, once, had a strongly patchy distribution in Europe with core zones in alpine and boreal regions in Lateglacial and Holocene, which only rarely provide a suitable climate for bark beetles (Stauffer *et al.*, 1999; Axelsson & Östlund, 2001; Latałowa & van der Knaap, 2006). In order to shorten dispersal time it must be of uttermost importance for beetles to be able to localise host trees with patchy occurrence. Landing in non-host habitats in mixed forest landscapes would be a waste of time and energy. Accordingly, the semiochemical information provided by non-host species has been shown to have a strong repellent effect on *I. typographus*.

4.2 Inhibition of pheromone attraction

Several compounds in deciduous non-host trees have been shown to elicit strong antennal responses in *I. typographus* (Zhang *et al.*, 1999; Zhang *et al.*, 2000), and to reduce the attraction to pheromone. In particular, a blend consisting of verbenone, C_6-alcohols, C_8-alcohols and *trans*-conophthorin has been shown to reduce the attraction to pheromone traps strongly. The reduction is dose dependent, and very low doses of *trans*-conophthorin act synergistically to almost shut of the attraction (Zhang & Schlyter, 2004; Schiebe, 2006). Not all of the components in the tested blend, however, have a clear non-host nature in an evolutionary perspective. C8-alcohols are found in the bark of *Betula* spp. and *Populus tremula* (Zhang *et al.*, 2000), but are also indicative for wood rotting fungi (Ziegenbein *et al.*, 2006). The strongly

antennal active spiroacetal *trans*-conophthorin occur in *Betula* spp. bark (Zhang *et al.*, 2000; Zhang *et al.*, 2002), but is also shown to be a pheromone of scolytide cone beetles from the genera *Conophthorus* and *Pityophthorus* (De Groot *et al.*, 1998; Dallara *et al.*, 2000; Miller *et al.*, 2000). (–)-Verbenone, on the other hand, is oxygenated from host (–)-α-pinene and the *I. typographus* pheromone component *cis*-verbenol, and can be found in increasing amounts in beetle galleries after colonisation (Leufven & Birgersson, 1987). It is assumed to act as a signal indicating an overexploited host, and is also an anti-aggregation pheromone in other bark beetle species (Wood, 1982a). All these compounds influence the beetles' behaviour during flight before landing. As such they help beetles to avoid unsuitable habitats or host trees, and reduce the exposure to predators while evaluating a tree after landing.

4.3 Inhibition at high beetle attack pressure

The effect of the repellent blend was tested at forest edges in Slovakia without the use of pheromones to attract beetles. The beetle population was large during the experiment, and tree mortality was high. We applied a pair-wise design of control and treated zones with three doses (Fig. 5A), which was replicated six times in total, distributed over four localities and two years. In both years we achieved a highly significant reduction of attacked trees in treated zones compared to control zones, although the protection was not absolute (Fig 5B). To demonstrate the repellent effect of the blend in more detail it would have been necessary to record the attack process continuously over time. However, treated but attacked trees were attacked later than the first attacked trees in the control zones (Paper I, Table 3). Conspecifics attracted to the arising pheromone plumes, overrunning the repellent signals, may then have switched their attacks to neighbouring trees within the protected zones (Schlyter & Anderbrant, 1989). Thus, the effect of the repellent blend may inhibit host finding by the beetles, as long there is no pheromone attraction present. The duration of the protection will also be dependent on the size of the beetle population. The more beetles, the higher the probability that some beetles will land on hosts in spite of an artificial non-host signal nearby, and thus initiate a pheromone producing attack that attracts even more beetles. Yet, the inhibition of the attraction to pheromone sources may be strong enough to delay the spread of the infestation to give the recorded reduction in attacked trees in the experiment. In additional, unpublished experiments in forest reserves in Sweden in 2008 we observed strong protection of treated plots in

some areas with heavy outbreaks (Fig. 6), although the protection could not withstand the high beetle populations in other areas.

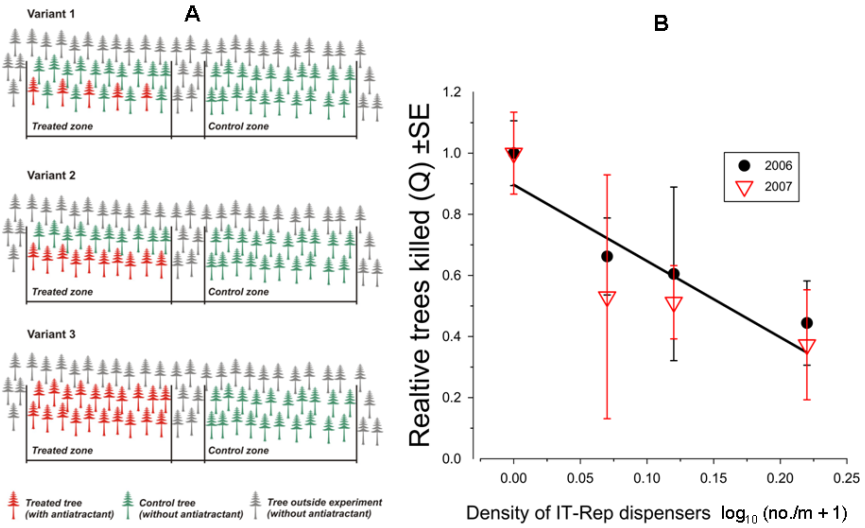
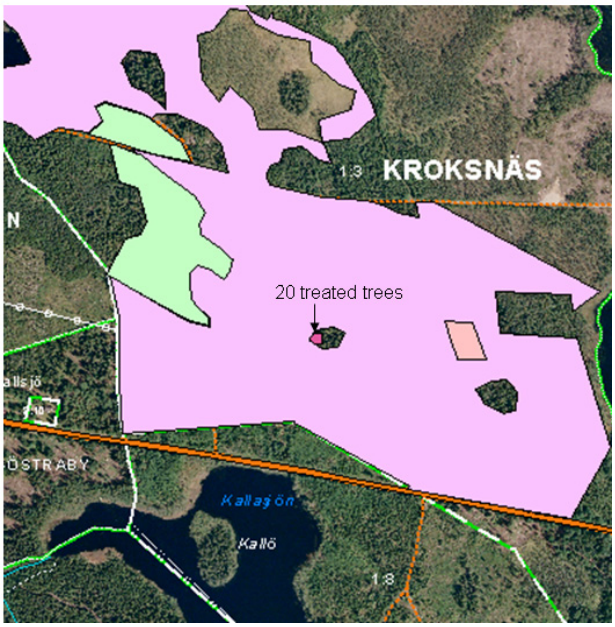


Figure 5. A) Design of the experiment testing a blend of NHV and verbenone at forest borders in Slovakia. (graph M. Blaženek) B) Dose–response of beetle-killed trees to the dose of anti-attractant dispensers in Slovakia summer 2006 and 2007. Response is the mean proportion Q of killed trees (trees killed in a zone/total killed in a pair of treated + untreated zones). Regression of arcsin √Q on log(dispensers/m forest edge +1) is shown (Paper I).

Figure 6. Aerial photograph of a nature reserve in Sweden, Kronoberg county, indicating the extension of *I. typographus* infestations in summer 2008. Grey area: beetle killed forest from 2007. Pink area: 75 – 95% killed trees (2008). Red spot: 20 trees treated with dispensers releasing NHV + verbenone were unattacked with an active inhibitory radius extending along the predominant wind direction. The map is kindly provided by Anton Holmström, Swedish Forest Agency.



4.4 Active inhibitory radius

In the Swedish experiment that corresponded the Slovakian design, the beetle populations in managed forest stands were markedly lower than in Slovakia. No trees in the treated zones and only one tree in control zones were attacked. The beetle populations were, however, high enough to result in lethal attacks within 90 m from treated trees in the three localities, indicating a high attack risk at the chosen sites. This pattern gave the possibility to estimate the active inhibitory range (AIR) of the non-host habitat signal. The highest numbers of attacks were found within 15 – 30 m from treated trees. Previous studies measuring AIR of single point sources in pheromone trap experiments gave estimates of 2 – 4 m (Zhang & Schlyter, 2003). In our case, the plumes from 20 dispensers at each treated plot indicating an unsuitable habitat seemed to divert dispersing beetles at least 15 m away from the plots. In contrast to previous pheromone trap experiments with single NHV release points, no attractive pheromone plumes existed, but there were unprotected host trees available in other directions with some, possible attractive effect. Therefore this study provides a more reliable estimate of NHV as a habitat signal. The study of background odour effects versus focus odours could be an interesting approach for future research.

4.5 Applications for forest management

The 'semiochemical-diversity hypothesis' of olfactory host recognition in mixed forest habitats, suggested by Zhang & Schlyter (2003), also provides a tool for protection of pure forest stands with little natural semiochemical diversity. The functionality of artificially applied semiochemical-diversity has been shown for different systems (Zhang & Schlyter, 2003; Jactel *et al.*, 2011; Schlyter, 2012). Repellent habitat signals do not, however, provide full protection of forest stands for long time. The demonstrated inhibition is most likely an effect of delayed landing rates that decrease the attack risks with a factor that probably is negatively correlated to population sizes. The delayed landing rates can be used to increase the attraction effect of pheromone traps, a strategy known as 'push and pull' (Cook *et al.*, 2007). This strategy should be used primarily in places with high hibernating local populations, e.g. during spring flight at attack spots of the previous year. A delay of attacks on exposed trees may also "buy time" for removing attacked trees before the spread of an infestation, in areas that are possible to keep under supervision. Urban forests of main importance for recreation and nature reserves may be core areas for this preventive forest protection tool.

5 Host discrimination (Paper II)

What are the incentives that impel a male *I. typographus* to land on a host in order to start colonisation? Olfaction may be only one of the drivers involved. Both visual cues (Niemeyer, 1985; Strom *et al.*, 1999), thermal and light conditions (Lobinger & Skatulla, 1996) may be of importance. It is generally agreed that physiological condition and fat content influences the dispersal capacities of bark beetles (Gara, 1963; Bennett & Borden, 1971; Thompson & Bennett, 1971; Botterweg, 1983). The response of *I. typographus* to pheromone cues and host material was correlated to the duration of dispersal and weight of the beetles in an extensive study by Gries (1985). Also for other *Ips* species responsiveness to both pheromones and host odours were related to lower fat content and desiccation (Hagen & Atkins, 1975; Gast *et al.*, 1993). The ability to discriminate between susceptible hosts and hosts with a high defence capacity should be an important factor influencing survival of pioneering males, regardless of other factors influencing landing in host habitats.

5.1 Chemical profile differences of constitutive host bark

To find possible biomarkers for susceptibility in Norway spruce we sampled trees in a forest with an epidemic beetle population before beetle flight had started. Out of 290 sampled trees, 19 trees were naturally attacked after sampling. Seven of these trees resisted the attack, whereas twelve trees were killed by the beetles. The comparison of the chemical profiles including 66 quantified compounds showed very few differences related to the beetles' host choice. The amount of bornyl acetate was significantly lower in the twelve successfully attacked trees than in adjacent control trees, that were not attacked by the beetles, and the seven surviving trees had significantly higher amounts of 1,8-cineole and (–)-limonene than the killed trees (Fig. 7). Generally, there

was no indication that the bark beetles had guidance by volatile host compounds included in this study when choosing trees to attack. Nor were there any differences between attacked and unattacked trees in non-volatile compounds, like phenolics and diterpenoids, that could have served as signs of susceptibility after landing. Bornyl acetate did not elicit any antennal responses in our GC-EAD recordings or in SSR (Andersson *et al.*, 2009), and is hence not a likely candidate for a behavioural indicator of susceptibility. The observed differences in 1,8-cineole and (–)-limonene between surviving and killed trees did not prevent a few pioneer beetles from trying to attack the surviving trees. However, it could be assumed, that higher amounts of these compounds may have decreased the pheromone attraction of conspecifics and thus prevented a mass attack.

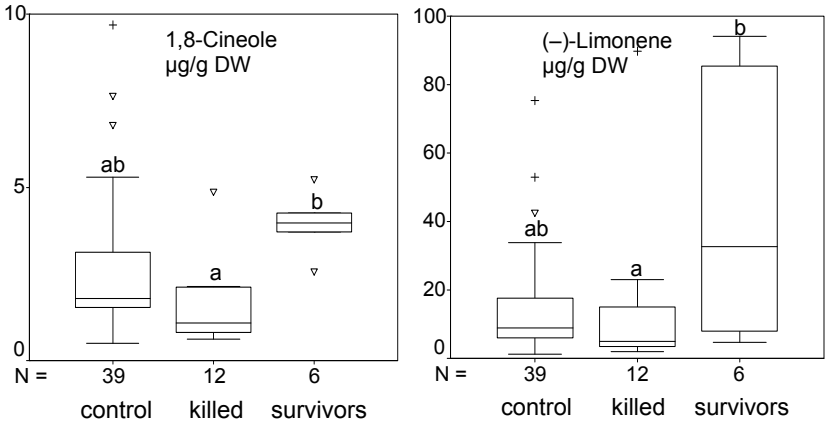


Figure 7. Absolute amounts of 1,8-Cineole and (–)-Limonene in constitutive bark of successfully and unsuccessfully attacked trees and unattacked control trees. Boxes show lower and upper quartile divided by the median, whiskers show minimum and maximum values and circles indicate outliers. Extreme values are not shown. Boxes with different letters are significantly different (ANOVA of log[x+1] transformed values with Tukey HSD and Dunnett's T3 for diterpenoids). (Paper II)

5.2 Differences in induced defence capacity

In order to assess the defence capacity of individual trees we applied a local treatment with methyl jasmonate (MeJ) near the base of each tree, the same day as constitutive samples were taken. MeJ is a phytohormone known to induce defence responses close to the treatment point (Krokene *et al.*, 2008). The induced area was sampled four weeks later. When comparing the chemical

profile of these samples by multivariate analysis of all included 66 compounds, six of the seven surviving trees grouped together, and were well separated from ten of the killed trees (Fig. 8A). Killed trees had a significant lower induction of most of the terpenes and four phenolics than surviving trees (Fig. 8B). They had also a very low induction of traumatic resin ducts in the young sapwood. Alkaloids decreased generally after MeJ treatment. No pronounced proportional changes in the chemical profiles occurred after induction with MeJ.

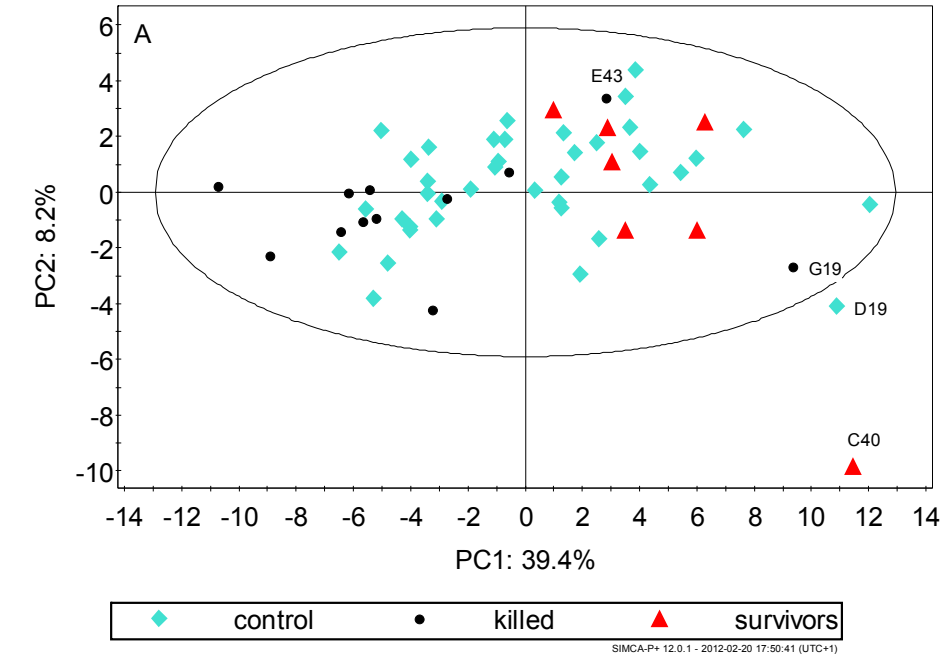
The observation of unsuccessful attacks on the surviving trees clearly shows that not all pioneers are able to discriminate between weak and strong defenders. We were, however, not able to track the detailed process of the failed colonisation attempts. Some of the unlucky attackers were found drowned in resin, while others seemed to have abandoned their attack holes in time. It is difficult to determine, if these beetles recognized their mistake by olfactory or gustatory cues or if they simply were physically pushed out by the resin. It could be observed, however, that not all of the abandoned holes were filled with resin.

5.3 The balance between tree defence and population size

Half of the unattacked control trees had chemical profiles matching either surviving or killed trees (Fig. 8A). If the beetle populations had been even larger than during our experiment, probably more of the putatively susceptible trees would have been attacked and killed. Yet, a second possible scenario could have obstructed the possibilities to detect differences between weaker and stronger trees: pheromone plumes from the first attacked, susceptible trees could have attracted so many beetles that even resistant trees nearby would have succumbed. Two of the killed trees illustrate this scenario: trees no E43 and G19 (Fig. 8A) had defences in the range of the surviving trees, but were, anyway, overcome by many attackers attracted by strong pheromone plumes released from neighbouring, attacked trees. E43 resisted, however, one week longer than its attacked neighbours, and G19 stood close to a tree ripped by lightning strike, and subsequently attacked. According to surveys by the Swedish Forest Agency over the whole region, there were higher trap catches the year of the experiment than the year before, but fewer attacks on standing trees occurred. One of the proposed explanations for this pattern is a higher tree vitality obtained after a wet late summer and autumn the previous year (Långström *et al.*, 2009). This pattern of higher trap catches and fewer attacks could be confirmed for our experiment forest, as the study reported in Paper I

was partly performed in the same forest and data for trap catches and killed trees were available for both years. The reported surviving trees and observed patterns in the chemical profile may, hence, be a result of a perfect balance between population size and host defence capacity, which rendered five small independent attack spots (Fig 9). If tree vitality had been lower, the attack spots might have grown larger at a few places, as fewer beetles would have been needed to overwhelm the trees' defences. In large attack spots it might be more difficult to discern the original focus trees targeted by the pioneers from trees infested as a result of spill-over of attacks from neighbouring trees undergoing mass-attack.

Figure 8. Principal component analysis of MeJ-treated *Picea abies* bark samples. A) Score scatter plot for the first and second components of all 58 MeJ-treated bark samples and 66 chemical variables. The first two components explain 47.6 % of the variance in the data. Samples lying outside the 95 % range of the Hotelling T2 ellipse, are considered to be outliers. B) Loading scatter plot showing the contribution of 66 chemical variables in explaining the distances along the component axes between the samples shown in (A). The longer the distance to the origin, the more important is a compound in explaining the position of a sample along either the x or y axis in (A). (Paper II)


A
E43
G19
D19
C40
PC2: 8.2%
PC1: 39.4%
control
killed
survivors
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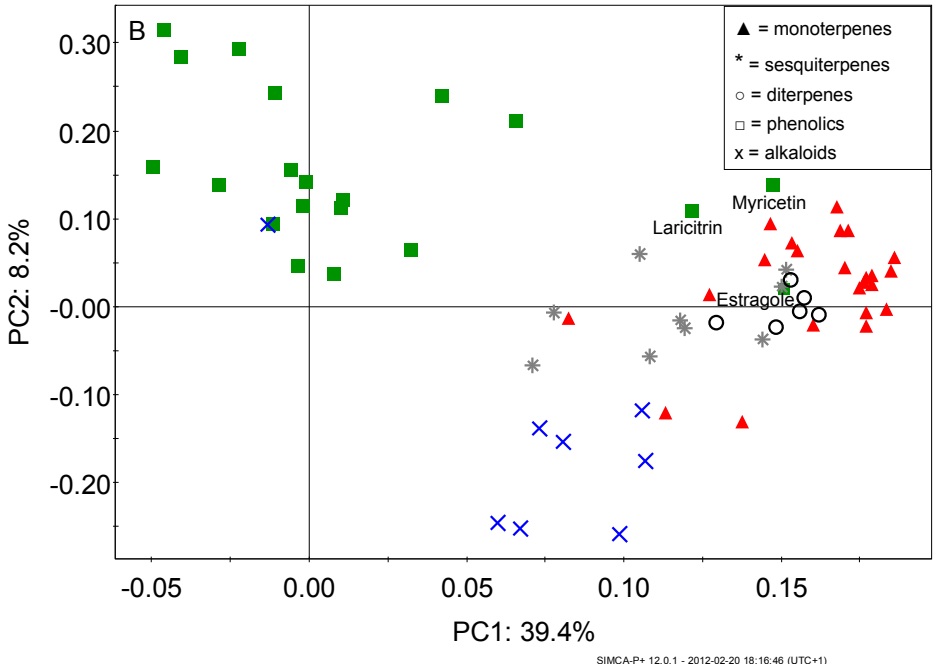
B
▲ = monoterpenes
* = sesquiterpenes
○ = diterpenes
□ = phenolics
x = alkaloids
Myricetin
Laricitrin
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PC2: 8.2%
PC1: 39.4%
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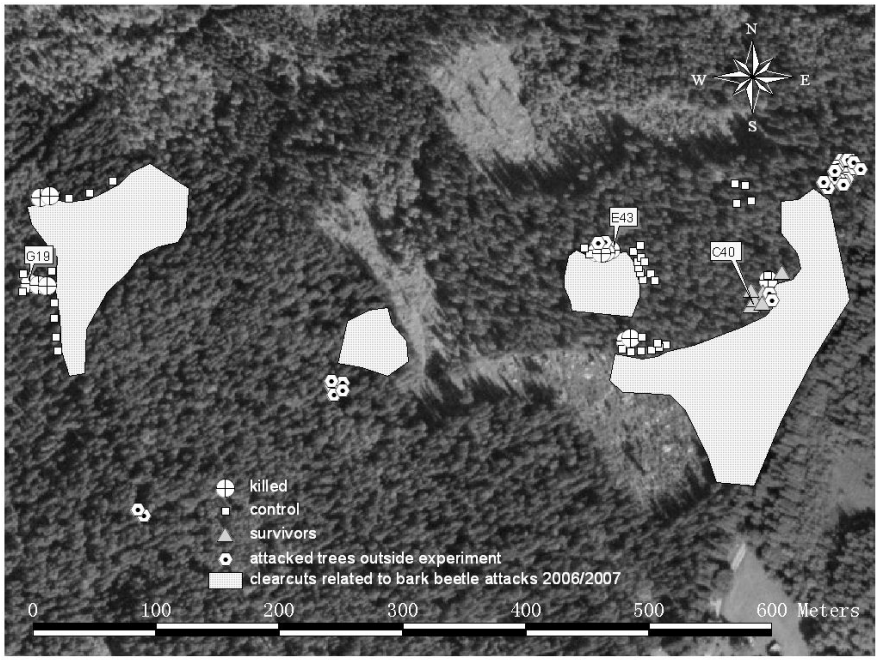


Figure 9. Aerial view of the study area in Parismåla, province Småland, Sweden. Marked areas were logged after heavy *I. typographus* attacks in 2006 and 2007. Experimental trees were sampled in spring before beetle flight. Of 290 sampled trees 19 were attacked during 2008. Attacked trees and 39 adjacent, unattacked controls were chosen for analysis in the study. Of the attacked trees, 12 were killed and 7 survived. Two killed trees (G19 and E43) that diverged from the main chemical pattern of killed trees in Figure 8A were excluded from further comparisons. Also one surviving tree (C40) with extreme amounts of analysed compounds in both untreated and treated samples was considered an outlier and was excluded. To give an overall impression of the extent of *I. typographus* attack in 2008, attacked trees that were not part of the study are also indicated (Paper II).

6 Recognition of host status (Papers III, IV)

The observation of similar chemical profiles of constitutive bark in attacked and unattacked trees (Paper II) indicate the lack for pre-attack host discrimination by pioneering *I. typographus* males. Nevertheless, there might be missing links in our knowledge about *I. typographus* host perception by means of olfaction or taste. Felled trees are the first choice for attacking beetles. They are missing any active defensive capacity, and the bark is usually still suitable for *I. typographus* colonisation the first summer after felling (Göthlin *et al.*, 2000). Thus, the volatile profiles from felled trees, being obviously highly susceptible, should be assessed for compounds eliciting antennal responses. In addition, can we detect differences between susceptible felled trees and healthy standing trees with regard to antennal active compounds?

6.1 GC-EAD studies of host volatiles

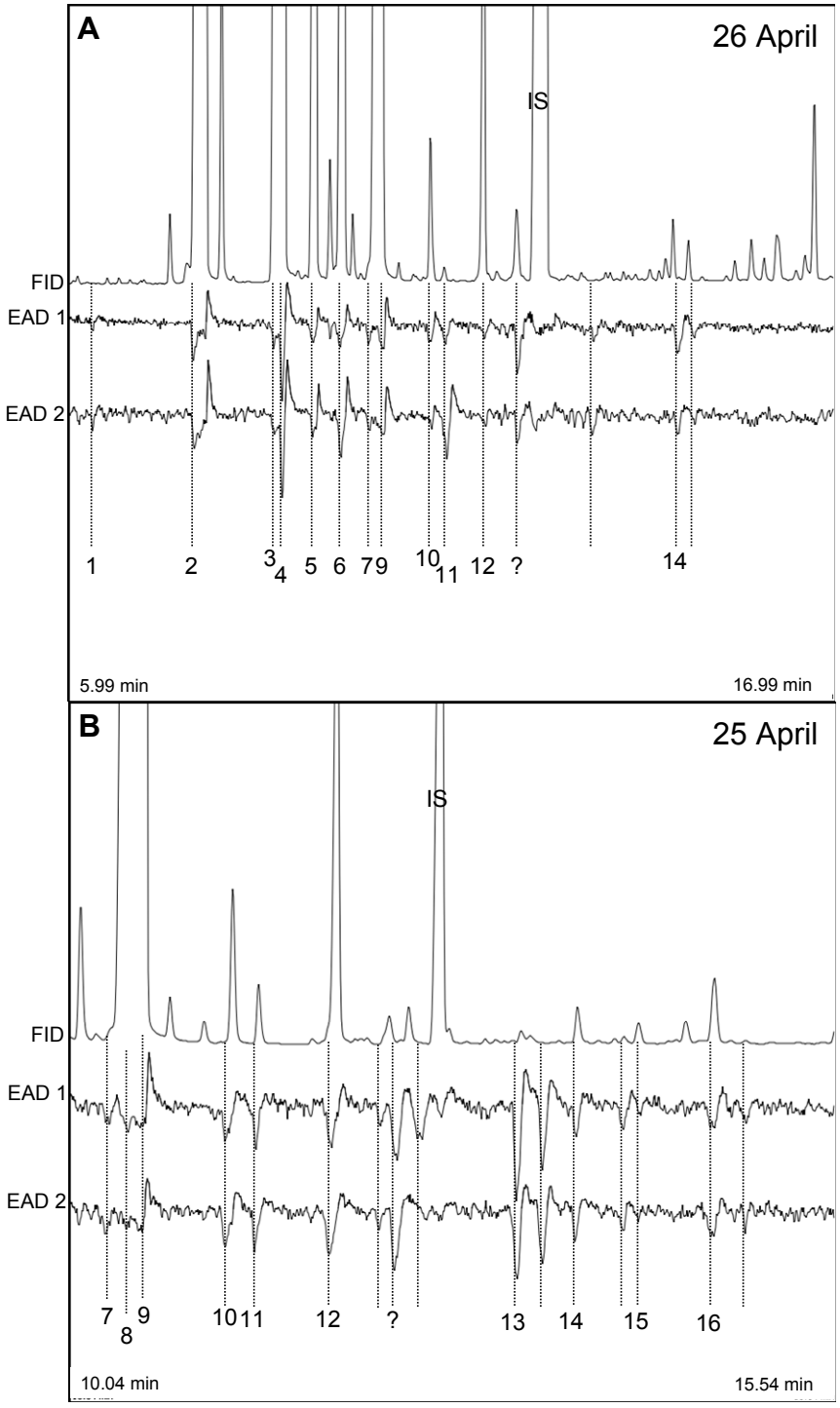
We collected volatiles from felled and standing trees from the start of beetle flight until felled trees were colonised, and on unattacked stem parts of colonised trees (Fig. 10). The biological activity in aeration samples was analysed by GC-EAD using beetle antennae (Fig. 2). Initial problems to achieve clear responses were overcome, when only beetles were used that had been stored for longer than one month in ≈ 3ºC and were well hydrated. Every sample was analysed on an equal number of beetles from each sex until at least four recordings with sufficient quality had been obtained. No differences in responses between sexes could been observed, consistently with earlier studies (Tømmeras & Mustaparta, 1987; Andersson *et al.*, 2009). The responses to the major terpene hydrocarbon constituents of *Picea abies* odour corresponded to the already known OSNs tuned to these compounds (Andersson *et al.*, 2009).

Also *para*-cymene and the oxygenated terpene 1,8-cineole, although only present in small amounts, regularly elicited responses, as previously shown by Andersson *et al.* (2010).

Another minor hydrocarbon constituent, the previously untested compound γ-terpinene, only occurring in small amounts in *Picea abies* bark, regularly elicited strong responses in the recordings. Responses to one of the most variable compounds in Norway spruce, 3-carene (see Paper II), showed some irregularity, when tested on different beetles using the same sample. Some question marks remained regarding the activity of sabinene, as the peak consisting of sabinene and β-pinene sometimes elicited a double response, whereas synthetic sabinene only elicited a single response.

The most important finding in the GC-EAD studies was, however, that several very small peaks, of which only camphor and estragole could be directly identified by mass spectra, elicited highly repeatable, very strong antennal responses. Other small peaks eliciting strong responses were under the identification level by GC-MS or co-eluted with other compounds. By extended screening of all samples and testing of synthetic candidates, we were able to identify responses to styrene, *trans*-thujan-4-ol, terpinene-4-ol, pinocarvone and putatively to pinocamphone and isopinocamphone. By testing synthetic compounds as candidates for unknown GC-peaks we found responses to α-terpineol, carvone and geranyl acetone, but these could not be matched to EAD responses to unknown peaks in aeration samples. A very strong response to a peak putatively identified as nonanal could not be verified by responses to the synthetic compound. Two typical GC-EAD chromatograms are shown in figure 11, and all tested compounds and their corresponding responses are shown in Table 1.

Figure 10. Bark volatile sampling from felled and standing trees 2009 in Parismåla. Volatiles were sucked from the open space provided by an aluminium grid below the foil through an adsorbent column for four hours. The columns were eluted with pentane/ether for GC-EAD studies and chemical analysis.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10.04 min
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Figure 11. GC-EAD recordings from one male and one female *I. typographus*. A) Example for the entire chromatogram from a sun exposed standing tree. B) Volatiles from a felled tree sampled at the day of first beetle attack. This graphs shows the part were oxygenated compounds elute on a HP5 column. 1) styrene 2) α-pinene 3) sabinene 4) β-pinene 5) myrcene 6) 3-carene 7) *p*-cymene 8) 1,8-cineole 9) γ-terpinene 10) *trans*-thujan-4-ol 11) terpinolene 12) camphor 13) pinocamphone 14) terpinene-4-ol 15) estragole ?) putatively identified to nonanal, but was not verified by the synthetic compound. IS) Internal standard heptyl acetate. Unmarked responses are not identified. Responses at the elution time of the standard are due to an unidentified co-eluted compound. Most recordings showed no response to the standard. (Paper III)

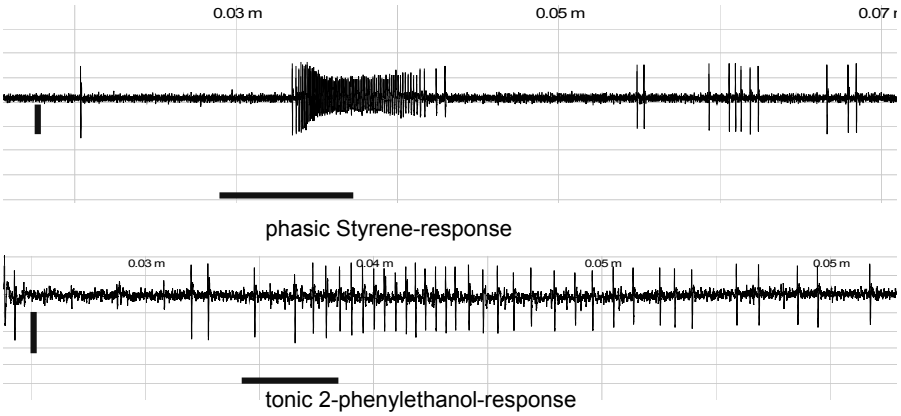

Figure 12. Response pattern in the OSN specifically responding to styrene and secondary to 2-phenylethanol. Both excitatory responses were after the highest dose (10µg) of a 0.5 sec stimulation pulse (horizontal bar). Vertical bars indicate 1 mV.

6.2 Single-sensillum recordings

After the detection of several previously unknown antennal active host compounds, we tried to localise corresponding OSNs with SSR-technique. The monoterpene γ-terpinene was found to activate a neuron, previously found to be tuned to *p*-cymene (Andersson *et al.*, 2009). This OSN-type regularly also responded with medium strength to estragole and carvone.

One OSN-type mainly responded to different host alcohols and ketones. Regularly it responded to terpinene-4-ol, α-terpineol, and the non-host compound 3-octanol. The strongest response was, however, elicited by *trans*-

thujan-4-ol. The dose-response test showed the strongest detected excitation at the 10 ng-level for any tested compound on any OSN-type. This compound could only be tested once, as it was identified late in the study. It elicited, however, regular strong responses in GC-EAD and can, thus, be regarded to be reliably verified. Terpinene-4-ol sometimes also elicited responses in other OSN-types.

An OSN-type regularly responding to camphor also responded with medium strength to (+)-α-pinene and to β-pinene. When pinocarvone was aquired late in the study, it elicited even stronger responses in this type than camphor. In some cases this OSN also responded with low or medium strength to other ketones or alcohols, and in three cases to estragole with similar strength as camphor. An OSN responding to camphor and pinocamphone in *I. typographus* has previously been identified by Tømmerås (1985). Therefore it can be suggested that this cell-type is responding to different ketones with similar configuration.

Styrene, identified late in the study, was found to elicit a strongly phasic response in an OSN-type, that also was elicited by 2-phenylethanol with a medium, explicitly tonic response (Fig. 12). All detected OSN ligands of host compounds and other compounds detected in this study for *I. typographus* are compiled in Table 1.

The SSR-studies led to indices of some genetic or physiologically conditioned variation. In many cases responses were only found occasionally and could not be repeated. On the other hand one or a few specific cell types often easily could be found many times when choosing randomly among sensilla on the same antenna, but could not be found at all when searching for it on another beetle antenna. Phenotypic plasticity within olfaction in a species with strong aggregative behavior may be an evolutionary mechanism allowing adaption to a highly variable host and fast shifts in population dynamics.

Table 1. Antennal activity of host compounds and some other compounds with antennal activity tested in Paper III

Compound	Origin	EAD-activity	OSN-activity	Compound	Origin	EAD-activity	OSN-activity
MT-hydrocarb.				**oxygen. MT**			
(−)-α-Pinene	H	+	+	1,8-Cineole	H	+	+
(+)-α-Pinene	H	+	+	*trans*-Thujan-4-ol‡	H	+	+
Camphene ‡	H	–	?	Camphor	H	+	+
Sabinene ‡	H	?	?	Pinocarvone*‡	H	+	+
β-Pinene	H	+	+	Myrtenol*‡	H	–	?
Myrcene	H	+	+	(Pinocamphone)	H	+	+
3-Carene	H	+	+	Terpinene-4-ol ‡	H	+	+
α-Terpinene‡	H	–	?	(Isopinocamphone) ‡	H	+	?
para-Cymene	H	+	+	Limonene oxides (*E*&*Z*)* ‡	H	–	?
Limonene	H	+	+	α-Terpineol*‡	H	+	+
β-Phellandrene‡	H	–	?	Verbenone*	H	+	+
γ-Terpinene‡	H	+	+	Carvone*‡	H	+	+
Terpinolene	H	+	+	Bornyl acetate	H	–	–
Other				Geranyl acetone*‡	NH	+	?
Nonanal‡	H	?	–				
2-Phenylethanol*‡	PH	+	+				
Styrene‡	H	+	+				
Estragole‡	H	+	+				

* Compound tested only with synthetic compound
\+ Reproducible or regular EAD activity to compound in aeration sample or synthetic compound
– No activity recorded to compound present in aeration sample or synthetic compound
? No reproducible or unverified activity
( ) Compounds in brackets are only putatively identified by match of mass spectra to Wileys library, other compounds are identified with mass spectra and by comparison of retention times and mass spectra with authentic standards. EAD activities are tested in natural aeration samples and synthetic standards.
‡ Compounds not earlier reported in litterature (Tømmeras & Mustaparta, 1987; Andersson *et al.*, 2009).
Origin: H = host; PH = pheromone component; NH = non-host

6.3 The role of oxygenated compounds in host chemistry

Most of the above described compounds that elicit strong responses, but only occur at very low proportions in host volatiles, are oxygenated monoterpenes. Oxygenated monoterpenes are derived from autoxidation, as well as microbial oxygenation of hydrocarbon precursors (Leufvén *et al.*, 1984; Hunt *et al.*, 1989; Lindmark-Henriksson *et al.*, 2004), and can also be products of induced monoterpene synthase activity in the tree, as e.g. linalool, borneol and α-terpineol (Martin *et al.*, 2003).

In order to assess the role of oxygenated terpenes in Norway spruce, all volatile samples were analysed with special emphasis on minor constituents. In addition, we included unused bark samples with MeJ-treated trees that had been saved from the 2008-study (Paper II). The analyses showed, that the oxygenated terpenes 1,8-cineole, pinocarvone, pinocamphone and α-terpineol attained higher proportions in the bark after MeJ-treatment. The proportions of several oxygenated terpenes in volatiles from felled trees also increased over time. Many studies assessing conifer defence responses concurrently found no clear changes in relative composition of oleoresin constituents, but pronounced quantitative changes of terpene hydrocarbons (Paper II; Raffa & Berryman, 1982a; Raffa & Berryman, 1982b; Raffa & Smalley, 1995; Martin *et al.*, 2002; Zhao *et al.*, 2010; Boone *et al.*, 2011). In contrast, the proportional changes found for oxygenated terpenes in our study, and also previously shown for MeJ treated foliage of *P. abies* (Martin *et al.*, 2003), make these compounds plausible candidates as indicators for host vitality. The strong responses to very low titres of them in host odours underline their biological importance for beetle host-perception.

6.4 Correlation of host volatiles to beetle attack

The absolute amounts of volatiles released from felled and standing trees, as well as proportions of single compounds were correlated to beetle attack. In addition to the planned experiment, a just started natural attack on standing trees was discovered near by the experiment trees, by chance, and could be included in the measurements. The release on felled trees was measured both near the cut end and in the middle of the crown. The trees released significantly higher amounts at mid crown, probably mostly due to thinner cork bark, but maybe also due to other differences in bark physiology and a higher terpene bark content (Baier *et al.*, 1999).

In the first two weeks of the flight season no attacks on felled trees occurred, while the observation pheromone trap > 100m from the felled trees had caught ≈ 200 beetles, when the first beetles were observed walking on felled trees. Four out of 34 passive landing traps, mounted on standing trees within 1 – 9 m to felled trees, started to catch a few beetles first two days after beetle colonisation on felled trees had started. During the following weeks, colonisation densities increased on the felled trees, while only single beetles were caught in passive landing traps. Two standing trees very close to a felled tree with high colonisation density were attacked, but resisted. A passive trap on one of these trees caught 43 beetles. During a very warm summer period five weeks after the first attacks occurred, one wind damaged standing tree and a neighbouring healthy tree were found attacked at ≈ 30 m from the felled trees. On two more healthy trees the firsts entrance holes could be observed. Aeration samples were taken immediately from the upper unattacked part of the stems and from neighbouring yet unattacked trees, of which two more were attacked during sampling. The next day four more trees were attacked during sampling, while eight sampled trees remained unattacked.

Comparisons of total measured volatiles from felled and standing trees showed, that the highest release rates were achieved from felled trees during time of first attack, while standing healthy trees had very low rates. Comparing standing trees under natural attack with adjacent unattacked trees showed that all attacked trees had release rates in the range of the felled trees, while most of the unattacked trees had lower rates (Fig. 13). Two similar studies, correlating emission rates or bark content of felled logs to bark beetle colonisation, consistently found, that trees with higher terpene hydrocarbon emission or bark content were attacked earlier and with higher densities by *I. typographus* (Reddemann & Schopf, 1996; Baier *et al.*, 1999).

Colonisation density on felled trees was significantly, positively correlated with increasing proportions of styrene, β-pinene/sabinene, 1,8-cineole, pinocamphone, pinocarvone and verbenone, and significantly negatively correlated with limonene/β-phellandrene.

The observed patterns of colonisation sequence and catches in passive landing traps correlated with released rates of host volatiles indicate that host volatiles are of importance for the initial host location, before arising pheromone plumes become the strongest attractant for dispersing beetles.

It is, however, extremely difficult to estimate the strength of plumes arising from individual or groups of trees that dispersing beetles may encounter. Our comparative measurements show the correspondence of release rates from point sources (≈ 5 dm^2 of bark surface) to the onset of beetle attack. It can only be speculated, how the released volatiles from the whole bark and foliage of

many trees interact within the natural context, and how the cacophony of attractant and interruptive signals spatially and temporally impact on beetle perception. For a discussion of similar attempts to estimate release rates and their impact on bark beetle orientation see Seybold et al. (2006).

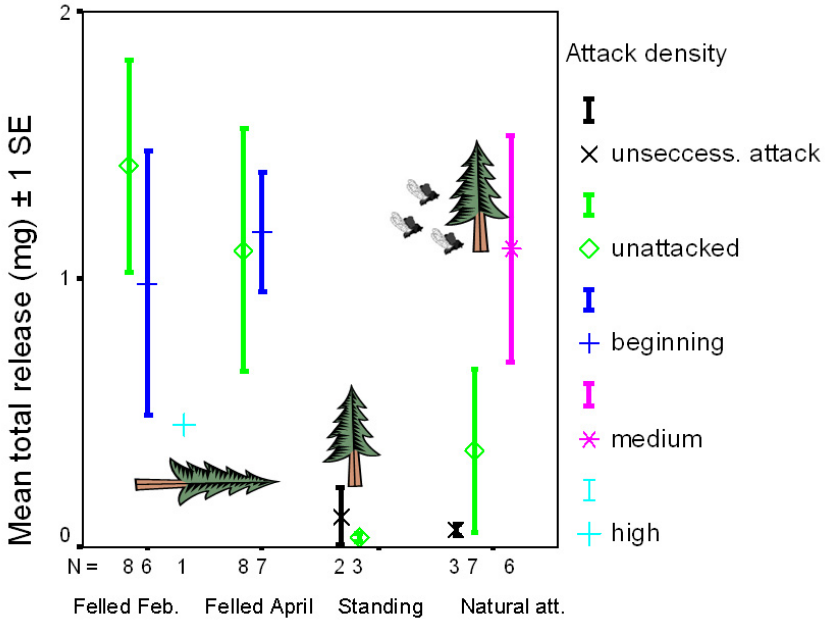

Figure 13. Means of total volatile release (per ≈ 5 dm^2 / 4 hrs) measured at mid crown from trees felled in February or April, at crown base from standing healthy trees and from standing trees under natural attack. Attack densities assign estimated densities of entrance holes: unsuccess. att: unsuccessful attack on two standing trees near by felled trees and on three trees within natural attack; eight unattacked trees within the group of natural attacked trees are control trees that remained unattacked.

6.5 Behaviour related to oxygenated host compounds

Small titres of oxygenated host compounds elicit considerably stronger antennal activity than the major monoterpene hydrocarbons. The strength of responses elicited to them are in the range of pheromone compounds and non-host volatiles, that provoke well established behaviours. To find the behaviour coupled to the antennal activity of single compounds may, however, be a difficult task, as specific ratios of the involved compounds may be the only entity eliciting a specific behaviour (Bruce *et al.*, 2005).

6.5.1 Trap experiments

A first attempt to find a behaviour to some of identified elicitors was the field test of a blend of oxygenated compounds. The blend was dispensed at three different doses together with a background of monoterpene hydrocarbons, without the interference of pheromones. We used release rates within the range of natural release by one tree in different state of degradation, calculated from our measurements. This experiment showed no attraction to host compounds different from a blank control trap.

The second field experiment tested the modulation of pheromone attraction by single compounds in two natural doses. We used low pheromone release rates in order to fashion a beginning attack, where pioneer beetle – host interactions may modulate the attraction of newcomers. This experiment showed a clear inhibition of both low and high dose of 1,8-cineole (Ci). The high dose of verbenone showed inhibition in similar strength, and other test compounds showed tendencies for both inhibition and amplification. The peripheral detection of Ci differs from other host compounds, as the excitation of OSNs tuned to Ci not only elicits a response from this neuron-type, but also inhibits the activity of an OSN, co-localized in the same sensillum, tuned to the essential pheromone component *cis*-verbenol (Andersson *et al.*, 2010). Although previously shown for higher doses, the inhibition of pheromone attraction by Ci at natural low doses confirms the importance of this compound for the interaction of host and pheromone perception. Verbenone was added as active control in the experiment, and its well established inhibiting effect (Bakke, 1981; Schlyter *et al.*, 1989) was confirmed.

6.5.2 Feeding assays

When beetles have landed on host trees either randomly or by previous kairomone attraction, gustatory cues may be more important than olfaction for the final decision to enter the tree or not. The search for behaviour related to the detected compounds with antennal activity was therefore further pursued by testing their modulation of feeding. The feeding of an artificial medium was enhanced by addition of a blend of host monoterpene hydrocarbons and abietic acid, which was applied for all further comparisons with addition of the test compounds, and also in all controls. The no-choice feeding assay in glass tubes with single beetles, previously developed by Faccoli *et al.* (2005; 2007), was modified by applying the test compound in several layers to achieve a more even distribution. In addition, this study tested compounds at relevant concentrations found in natural host bark, varying between low concentrations (susceptible trees) to high concentrations found in resistant trees (Paper II).

In contrast to electrophysiological responses, the behavioural effect in feeding differed between sexes. One could expect, that males, initiating attacks, should be more responsive to host signals. Consistently with Faccoli *et al.* (2005; 2007), the factor sex was significant for estragole, verbenone, and camphor, and the effect was stronger for males than for females, except for the phenolic estragole.

Verbenone was added as positive control also in this assay. It exhibited a weak, but clearly dose dependent inhibition on males. Inhibition on male feeding by Ci, terpinene-4-ol and camphor was dose dependent only for low and medium dose, but the highest dose had no effect. The same pattern, but in the opposite direction could be observed for carvone on males. This effect of increased feeding in males was not strong, but could interestingly be observed also in the trap experiment, were carvone had a tendency to increase pheromone attraction in the higher dose. In contrast, Faccoli *et al.* (2005) found a clear inhibition of feeding by carvone, but they used a minimum concentration 50 times higher than the maximum concentration used in our study. In addition, they added the test compound on the top of the medium, most likely giving an even higher concentration in the upper part of the tube.

Our results indicate compound specific, physiological thresholds. The contrasting effects of different doses and different compounds indicate the complexity in the behaviour evoked by the bouquet of host odour. In addition, the effect may differ not only between sexes, but also between beetles in different physiological condition, like different fat content after hibernation or maturation feeding, or between beetles from low density endemic or high density epidemic populations (Hagen & Atkins, 1975; Anderbrant *et al.*, 1985; Nemec *et al.*, 1993; Bohlander & Schopf, 2000 ; Sallé *et al.*, 2005a; Sallé & Raffa, 2007).

7 Future prospects and applied aspects

The Eurasian spruce bark beetle is one of the major pest insects in European managed forests. Climate change is expected to accentuate disturbances like storm felling and drought. Higher temperatures and prolonged seasons will probably increase reproduction rates of this insect, mainly due to changed voltinism (Jönsson *et al.*, 2009). Range expansion into regions that not previously have experienced outbreaks may be another increasing threat for boreal forest ecosystems.

Solutions for sustainable forest management in a changing environment should include aspects of general increased tree health in managed forests, and new operational methods for pest management. Fast growing forests and forests in pure stands show lower defence characteristics than trees in mixed stands with lower radial growth indices (Baier *et al.*, 2002). Pure stands are more exposed to herbivory by specialist herbivores (Jactel & Brockerhoff, 2007; Kausrud *et al.*, 2012), but in extensive areas of planted conifer forests no fast changes are feasible in order to increase semiochemical diversity. The artificially applied semiochemical diversity tested in this thesis can be developed further to include integrated 'push and pull' systems, cheaper chemicals and area wide distribution (Gillette *et al.*, 2012; Schlyter, 2012).

But to gain a more complete understanding of bark beetle – conifer systems even more basic research is needed. The interactions of bark beetles and their symbiotic fungi are still poorly understood. Strong olfactory responses to putatively fungi produced compounds need an explanation. An assessment of multipartite symbiotic relationships with fungi and bacteria possibly provide new strategies for pest control, involving manipulations of symbiotic associations (Popa *et al.*, 2012). An important gateway to understand beetle host choice mechanisms could be to explore the impact of beetle physiological condition on beetle olfaction. As physiological condition is related to

colonisation density (Anderbrant & Schlyter, 1989) and thus to population dynamics (Komonen *et al.*, 2011), shifting attack preferences could possibly be linked to different host perception. The detection of the strong olfactory activity elicited by stress related host compounds is a promising link to an improved understanding of host choice in *I. typographus* and possibly other aggressive bark beetle species. Comprehensive behavioural studies will be necessary to find the relevant behaviour. Improved pheromone attractants and improved repellents for use in pest management are possible applications for knowledge derived from these studies.

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Acknowledgements

All through my childhood I was filled with a strong interest in nature. Yet, I started my career in biology by refusing a place at the well reputed University in Tübingen in 1978. The practical part of me was crying out for learning nature by doing. Thus, for the first part of my adult live, I was engaged with learning the most essential part of human culture: “agri-culture”. I finally became a gardener in Småland, and run a farm producing all kinds of organically grown vegetables. To survive as a farmer nowadays, your business has to grow continuously. Consequently, my project to learn nature by doing was changed more and more into administration of a growing workforce, repairing equipment, and the rush of deliveries. I had to give up my project, and decided to take up studies again. I applied for a second time, now at Kalmar University. It would perhaps have been easier to enter into science, when I was younger, than when I was a father of teenagers, and never had touched a computer.

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